Nodulation and root growth of forage legumes sown into tall fescue swards

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Abstract

Establishing forage legumes into endophyte-infected tall fescue (*Festuca arundinacae* Schreb.) pastures is problematic, especially in well-established stands. A oversowing field experiment determined if this problem was because of poor nodulation. Four renovation techniques, clipped sward (treatment A), herbicided + rye seeding in the previous autumn (treatment B), herbicided in the autumn and spring (treatment C) and herbicided to suppress the sward (treatment D), were investigated to determine their effect on nodulation and root growth of lucerne (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) and white clover (*T. repens* L.) at 16, 22 and 29 d after sowing the legumes. A pot experiment was also conducted under optimal growth conditions and using the same soil to determine the nodulation and root growth potentials of these legume species. At adequate rhizobial populations ($>6 \times 10^4$ cfu g^{-1} soil), substantial nodulation of all species occurred by 29 d after sowing in treatments C and D, whereas nodulation of clovers was usually reduced in treatment A. Total root lengths for all sampling dates, species and treatments were severely restricted, especially under treatment A. A general correspondence of nodulation with root growth was observed for all species, with high correlations ($r \geq 0{\cdot}85$) between these variables for all legume species and treatments, suggesting that soil moisture, and possibly competition for light, were the limiting factors. These results demonstrate that weak stands of forage legumes, typically found when sown into tall fescue swards, are probably not because of inadequate nodulation. Rather, inhibition of root growth by detrimental physical/chemical conditions or allocation of limited photosynthate to shoots instead of roots is suggested.

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Introduction

Pastures dominated by tall fescue (*Festuca arundinacae* Schreb.) are often deleterious to livestock performance because of toxins produced by association with the endophyte, *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin comb. nov. One means for overcoming this problem is to include legumes (Hoveland *et al.*, 1981; Coffey *et al.*, 1990; McMurphy *et al.*, 1990; Chestnut *et al.*, 1991) in the sward. However, introducing legumes into tall fescue swards is difficult, particularly in well-established and endophyte-infected stands (Fribourg *et al.*, 1978; Luu *et al.*, 1982). Numerous attempts have resulted in weak legume stands in such swards, despite using the best management practices available for no-till or interseeding (oversowing) renovation (C.S. Hoveland, J.P. Fontenot, D.P. Belesky, pers. comm.). Also, pastures dominated by *Neotyphodium lolii* (Latch, Christianson and Samuels) Glenn, Bacon, and Hanlin comb. nov.-infected perennial ryegrass (*Lolium perenne* L.) tended to have less white clover (*Trifolium repens* L.) than non-infected pastures (Watson *et al.*, 1993). Additionally, Quigley *et al.* (1990) demonstrated that radicle growth of white clover and subterranean clover (*T. subterraneum* L.) was affected in the same manner as the field-grown plants when grown in water extracts from infected or non-infected perennial ryegrass herbage.

Determining the cause of this problem can be approached from either a soil perspective (e.g. because of mineral toxins, allelopathy, or soil moisture content) or from a developmental and functional perspective (e.g. because of poor legume nodulation or N_2-fixation). In an earlier tall fescue renovation experiment, poor nodulation of lucerne (*Medicago sativa* L.) and several lespedeza (*Lespedeza cuneata* L., *Sericia lespedeza L.*) species, oversown into a herbicide-treated tall fescue sward, was observed (D.P. Belesky and E.L. Mathias, unpubl. data). Because of this, and because no

literature exists on forage legume nodulation under established tall fescue, much less than under endophyte-infected tall fescue, investigating symbiosis establishment with rhizobia seemed imperative. Further, as seedlings are particularly likely to show inhibitory effects, it is important to examine symbiosis establishment as early as practically possible. Determining this, however, is not a trivial matter because of the uncertainty of assuring saturation levels of infective rhizobia in soil systems over the course of an experiment, especially at the field scale (Staley, 2003).

Vigorous, volunteer white clover growth was observed in an area, adjacent to the present study site and renovated 4 years earlier with black oats (*Avena strigosa* L.), where whole-plant oat residue was retained on the soil surface over winter, followed by rotovating the following spring. It is possible that decomposing residues (tall fescue and/or oats) may have mitigated the deleterious soil conditions associated with the previous tall fescue sward (Matthews and Clay, 2001). To determine if renovation techniques affect establishment of forage legumes, by virtue of altering nodulation (early symbiosis establishment), field plots during the establishment year of a long-term tall fescue renovation study were used. Nodulation of lucerne, red clover (*T. pratense* L.) and white clover, following the application of the four sward treatments, was monitored, as well as legume root growth and development.

Materials and methods

Research was initiated in the autumn of 2000 on a USDA-ARS farm in southern West Virginia, USA (37°47′55″N, 80°58′19″W; altitude, 893 m; aspect, S35°W; slope, 10%). Soil at the study site belonged to the Clymer channery loam series (fine loamy, mixed, mesic Typic Hapludult). Initial plant cover consisted almost exclusively of endophyte-infected, tall fescue that had been managed as a hayfield and cattle pasture for >30 years. Soil test results indicated adequate pH and major plant nutrients (Table 1).

Soil ('Study') cores at depths of 0–5, 5–10 and 10–15 cm were collected on 12 September 2000, just outside the treatment areas (corners of blocks), sieved to <2 mm and air-dried for chemical analyses. Identical sampling was done on 9 April 2001 for only the 0–5 cm layer and stored in the fresh condition at 5°C for determination of native rhizobial populations. Most probable numbers (MPNs) were determined according to Weaver and Frederick (1982), using seedlings grown for 21 d in a growth chamber (light:dark regime of 16:8 h at an average irradiance of 267 μmol m^{-2} s^{-1} at 25 and 22°C respectively, and 60% relative humidity).

Three blocks (10·1 m × 11·0 m; 0·9 m alleys) accommodated the four renovation treatments of the initial sward: treatment A, tall fescue clipped to a height of 2·5 cm just prior to legume seeding; treatment B, sprayed with glyphosate [*N*-(phosphono-methyl)glycine] (7·01 L ha^{-1}), rotovated and broadcast-seeded with cereal rye (67 kg seed ha^{-1}) in the previous autumn; treatment C, sprayed with glyphosate (7·01 L ha^{-1}) in the previous autumn and again in mid-April; and treatment D, sprayed with gramoxone (1′,1′-dimethyl-4,4′-bipyridinium dichloride) (3·51 L ha^{-1}) in mid-April. Treatments B and C were made in October 2000 to allow (approximately 6 months) decomposition of tall fescue residues. Experimental design was a split–plot, with legume species as main plots (3·0 m × 10·1 m) and treatments as subplots (1·8 m × 3·0 m), with three replicates.

On 30 April 2001, white clover, cv. Grasslands Huia, red clover, cv. Cinnamon, and lucerne, cv. Alfagraze, were interseeded, after inoculation with commercial rhizobia (Nitragin AB) according to the manufacturer's

Table 1 Chemical properties of Clymer channery loam soil used in the study*.

		Exchangeable cations (cmol kg^{-1})							
Depth (cm)	**pH_w**	**Ca**	**Mg**	**K**	**Na**	**Al**	**H**	**CEC†**	**Base saturation (%)‡**
Study soil									
0–5	6·83	4·85 (62·7)§	2·25 (29·1)	0·60	0·01	0·05	0·00	7·74	99·4
5–10	7·14	2·90 (62·0)	1·45 (31·0)	0·31	0·03	0·05	0·00	4·68	98·9
10–15	7·19	2·29 (62·1)	1·17 (31·7)	0·21	0·03	0·05	0·00	3·69	98·6
Control soil									
0–10	6·21	3·42 (67·3)	1·21 (23·8)	0·37	0·04	0·03	0·01	5·08	99·2

*All values are given on an oven-dry (105°C) basis; the 'Study' soil was collected on 12 September 2000 and the 'Control' soil was collected on 5 September 2001.

†CEC = Σ(Ca + Mg + K + Na + Al + H).

‡% base saturation = [Σ(Ca + Mg + K + Na)/CEC] × 100.

§Values in parentheses are % CEC.

instructions (Lyphatech, Milwaukee, WI, USA)[1], with a no-till seeder (Tye Pasture Pleaser, Model 104, Lockney, TX, USA) on 20·3 cm rows, equivalent to 5·6 and 11·2 kg seed ha^{-1} for the clovers and lucerne respectively. To maximize the likelihood of saturation levels of rhizobia over the course of the experiment, soil inoculations were made at 2 and 9 d after sowing. Commercial peat-based inoculum was suspended in distilled water, just before application, at a rate sufficient to provide at least $1{\cdot}8 \times 10^6$ colony-forming units (cfu) m^{-1} of row. Suspensions (100 mL) were applied directly on the surface of each of fifteen slit rows (each 2 m in length) per subplot in a 2·54 cm band by use of wooden troughs, open at the bottom.

At 16, 22 and 29 d after sowing, eight soil/root cores were taken from each subplot, equidistantly spaced along the slit row, to provide whole root systems for assessment of nodulation and root growth of legume seedlings. Core sizes were increased as follows: 16 d after sowing, 2·5 cm diameter, 5 cm length; 22 d after sowing, 4·0 cm diameter, 7·5 cm length; and 29 d after sowing, 6·5 cm diameter, 10 cm length. Intact cores were immediately brought to the laboratory and frozen. Upon thawing, roots were removed at the crown, thoroughly cleansed, and then preserved in 50% (v/v) ethanol. Eight roots were selected for analysis, based on their average appearance and structural integrity. Mature and adolescent (at least half-domed) nodules were counted on the entire root system without staining. Root images were acquired with an Epson Expression 636 Scanner (Epson America, Inc., Long Beach, CA, USA) and analysed with MacRHIZO, Version 3.10, software (Regent Instruments, Inc., Quebec, Canada) for total root length (TRL) and average diameter.

Soil samples for rhizobia assessments were also taken on 4 June 2001, 5 d after the 29 d after sowing sampling for soil and root cores (25 d after last inoculation). Eight cores (1·9 cm diameter and 5 cm length) were taken from each subplot within the inoculation band and pooled. Samples from each replicate were pooled by treatment (12 samples, total), sieved (<2 mm) and stored at 5°C until analysed as previously described.

A pot experiment under near-optimal environmental conditions (i.e. identical to those described above for growth chamber MPNs) was also carried out using 'Control' soil from an area, adjacent to the study site and that had been renovated with black oats 4 years earlier, and which had a healthy stand (approximately 95% cover) of volunteer white clover. The 0–10 cm layer was sampled on 5 September 2001, sieved (<4 mm), and used 12 d later in the fresh (never air-dried and stored at 5°C) condition. Pots containing 278 g soil (air-dry basis) were seeded, after seeds were inoculated with commercial rhizobia, and placed in the growth chamber. Immediately after germination, seedlings were thinned to rows at densities comparable to those found in the field study (approximately 1 cm^{-1}). At 2 and 9 d after sowing, plants were again inoculated by drenching at rates comparable to the field study. Plants were maintained at a soil moisture content of −0·03 MPa by daily, manual watering. Roots from two representative plants were harvested at 16, 22 and 29 d after sowing, then processed and assessed as described previously for field roots. Nodulation potentials (Np), as well as root growth potentials (Rp), represent the ratio of field to pot values × 100 (i.e. % maximum).

[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Statistical analyses

Analysis of variance (ANOVA), least significant differences ($LSD_{0{\cdot}05}$) and linear correlations were determined in SAS System for Windows, Release 8·0 (SAS Institute, Inc., Cary, NC, USA). Unless otherwise stated, differences were determined at the $P \leq 0{\cdot}05$ level of significance.

Results and discussion

Soil chemical properties, precipitation, and rhizobial populations

Soil from the study site (essentially the Ap horizon), prior to initiation of the experiment, had a near neutral pH (6·8–7·2), and contained high concentrations of basic cations and low concentrations of Al (Table 1). All exchangeable cations consistently decreased with depth to 15 cm. Plant-available P (resin inorganic P, plus bicarbonate inorganic P; Hedley *et al.*, 1982) was 165 mg kg^{-1} dry soil. These properties identify the soil as being of moderate to moderately high fertility and reflect its previous management history. As anticipated, the 'Control' soil was nearly identical in pH and exchangeable cations to that at the study site (Table 1).

Precipitation before and during the first half of the field experiment was below average. For 2 weeks prior to sowing and until 7 May (6 d after sowing), only 0·3 mm precipitation was recorded at the site. At 6, 10 and 14 d after sowing, precipitation of 11, 2 and 8 mm was recorded respectively. There was a 42 mm precipitation deficit, relative to the last 10-year average, for the interval from sowing until 16 d after sowing.

Precipitation during the second half of the experiment was above average. Between 16 and 29 d after sowing, 65 mm precipitation above average was recorded. However, precipitation was poorly distributed. Precipitation deficit occurred when the growth rate of cool-season grasses should be near maximum (Belesky and Fedders, 1995).

Native rhizobial populations in the 0–5 cm soil layer were approximately 10^3 cfu g^{-1} dry soil for the clovers, and essentially absent for lucerne (<10^1 cfu g^{-1} dry soil). These populations are considered adequate for clovers, but inadequate for lucerne (Wacek, 1997). At the end of the experiment, populations for all three legume species in soil from the slit-rows were substantially increased (Table 2), exceeding 6×10^4 cfu g^{-1} dry soil for all species and treatments. These populations exceed those required for adequate nodulation of each legume species in the experiment.

Nodulation of legumes

Consistent trends of increased nodulation (number of nodules plant^{-1}; Table 3) and TRL (cm plant^{-1}; Table 4) over time for all legume species and treatments, except for nodulation of white clover in treatment C, suggest that the methodology used for field sampling and laboratory assessments was sound.

ANOVA revealed significant main effects of both legume species and treatment on nodulation. However, no significant interactions between species and treatment were found.

Treatment affected alfalfa nodulation (number of nodules plant^{-1}) at 16 d after sowing, being two- to threefold greater under treatment D than under the other treatments (Table 3). There were no significant treatment effects after this time for lucerne. Nodulation of red clover was significantly increased by treatments B, C and D at 16 d after sowing, compared with

Table 2 Effect of various sward renovation treatments on soil rhizobial populations for various forages at the end of the experiment.

	(cfu $\times 10^5$ g^{-1} dry soil)		
Treatment*	**Lucerne**	**Red clover**	**White clover**
A	1·35 (0·4–4·5)†	0·76 (0·2–2·5)	5·57 (1·7–18·0)
B	0·58 (0·2–1·9)	1·39 (0·4–4·6)	5·76 (1·7–19·0)
C	1·21 (0·4–4·0)	2·20 (0·7–7·3)	7·78 (2·4–26·0)
D	1·08 (0·3–3·6)	ND‡	1·59 (0·5–5·2)

*Treatment A, clipped; treatment B, herbicide + rye; treatment C, herbicide only; treatment D, suppressed.
†Confidence limits at $P > 0.05$ level.
‡ND, not determined.

Table 3 Effect of various sward renovation treatments on nodulation of various forage legumes oversown into tall fescue.

		Nodulation at days after sowing (number of nodules plant $^{-1}$)		
Forage species	**Treatment***	**16**	**22**	**29**
Lucerne	A	1·29^{b}†	3·13^{a}	5·67^{a}
	B	0·96^{b}	4·71^{a}	8·00^{a}
	C	0·83^{b}	5·46^{a}	5·83^{a}
	D	2·79^{a}	4·08^{a}	4·88^{a}
Red clover	A	0·88^{b}	2·67^{a}	6·67^{c}
	B	3·38^{a}	5·33^{a}	9·00^{bc}
	C	4·04^{a}	5·58^{a}	13·5^{a}
	D	3·58^{a}	5·38^{a}	9·71^{b}
White clover	A	0·79^{a}	2·00^{b}	4·71^{a}
	B	2·17^{a}	2·67^{ab}	4·96^{a}
	C	2·58^{a}	2·50^{ab}	5·92^{a}
	D	3·08^{a}	3·38^{a}	6·33^{a}

*Treatment A, clipped; treatment B, herbicide + rye; treatment C, herbicide only; treatment D, suppressed.
†Values within a species column followed by the same letter are not significantly different ($P > 0.05$) as determined by the LSD method.

Table 4 Effect of various sward renovation treatments on total root length of various forage legumes oversown into tall fescue.

		Total root length at days after sowing (cm plant^{-1})		
Forage species	**Treatment***	**16**	**22**	**29**
Lucerne	A	11·9^{b}†	17·0^{b}	26·1^{c}
	B	13·2^{ab}	20·7^{b}	30·9^{bc}
	C	15·2^{a}	32·2^{a}	48·9^{a}
	D	14·9^{a}	17·0^{b}	34·3^{b}
Red clover	A	8·85^{b}	11·8^{c}	23·9^{c}
	B	10·9^{b}	18·7^{b}	28·1^{c}
	C	17·0^{a}	27·0^{a}	61·1^{a}
	D	14·9^{a}	24·1^{a}	39·1^{b}
White clover	A	5·12^{b}	6·93^{c}	11·8^{b}
	B	7·42^{a}	8·36^{bc}	11·3^{b}
	C	8·74^{a}	9·14^{ab}	20·4^{a}
	D	8·70^{a}	10·5^{a}	21·3^{a}

*Treatment A, clipped; treatment B, herbicide + rye; treatment C, herbicide only; treatment D, suppressed.
†Values within a species column followed by the same letter are not significantly ($P > 0.05$) different as determined by the LSD method.

treatment A. Similar responses, although not significantly different for most comparisons, were evident at 22 and 29 d after sowing. Nodulation response patterns of white clover to treatments generally mimicked those for red clover. Although no significant differences were found for white clover at 16 or 29 d after sowing, nodulation at 22 d after sowing was higher on treatment D than treatment A.

In the pot study, nodulation (number plant^{-1}) at 16, 22 and 29 d after sowing was, respectively, 4·0, 7·0 and 7·5 for lucerne, 5·0, 6·0 and 18 for red clover and 5·0, 5·5 and 8·5 for white clover. Nodulation potentials (Np) of the field-grown legumes by 29 d after sowing ranged, over all treatments, from 65–107% for lucerne, 37–75% for red clover, and 55–75% for white clover. Lowest Np values for both clovers were consistently found at all sampling times in treatment A. Thus, in treatments where normal soil moisture stress was not compounded by a growing companion grass, as was likely in treatments A and B, all field-grown species were well nodulated (Np = 54–78%) by 29 d after sowing.

Taken together, these results demonstrate substantial nodulation for all legume species under the herbicide treatments, and usually less nodulation when rye was sown after herbicide treatment, especially for the clovers. Clipping the sward typically resulted in the poorest nodulation for all legume species.

Legume root growth and development

ANOVA revealed significant main effects of both legume species and treatment on TRL, as well as a significant interaction between them. Based on the supposition of seed-size influences, subsequent analysis showed the interaction was not between red clover and lucerne but between white clover and the other two species, which did not differ.

For lucerne, treatments affected TRL (cm plant^{-1}) at each sampling, with differences primarily found between treatment A and treatments C and D (Table 4). For red clover, TRL was increased at all three samplings in treatments C and D compared with the other treatments. Response patterns of white

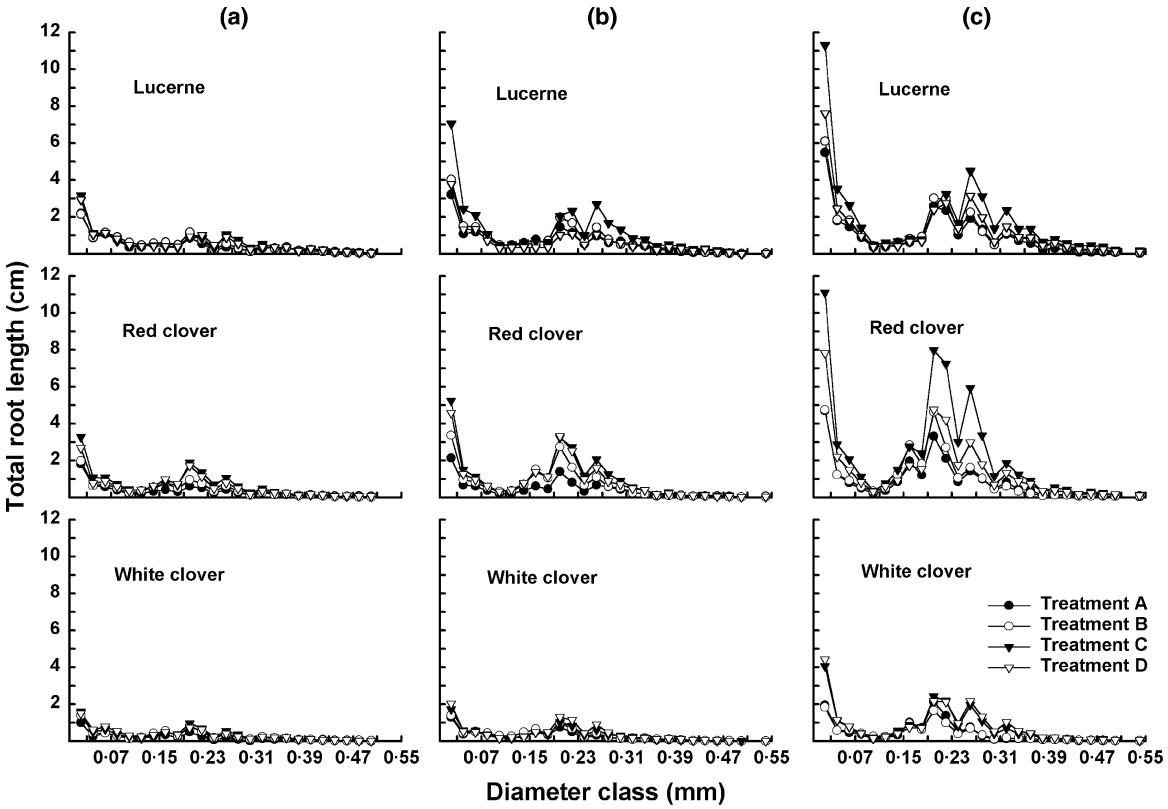

Figure 1 Sward renovation treatment effects [treatment A, clipped (•); treatment B, herbicide + rye (○); treatment C, herbicide only (▲); treatment D, suppressed (△)] on total root length, sorted by diameter class, of lucerne, red clover and white clover roots at (a) 16, (b) 22 and (c) 29 d after sowing in the field experiment.

clover TRL to treatments were similar to those for red clover, although responses under treatments C and D were not as consistently different from the other treatments.

In the pot study, TRLs (cm plant^{-1}) at 16, 22 and 29 d after sowing were, respectively, 104, 176 and 184 for lucerne, 101, 131 and 284 for red clover and 35, 43 and 72 for white clover. Root growth potentials (Rp) of field-grown legumes over all treatments by 29 d after sowing ranged from 14–27% for lucerne, 8–22% for red clover and 16–30% for white clover. Lowest Rp values for all species were nearly always found in treatment A. These results show that field root growth of all legumes examined was greatly restricted, especially in treatment A. Although low Rp values would be expected because of significantly different field and chamber growth conditions (essentially, optimal in the latter), the magnitudes of the differences are nonetheless striking.

When field-grown roots were sorted by diameter class (Figure 1), patterns of development for all three species were the same, generally, at 16 d after sowing. By 22 d after sowing, divergent patterns were apparent among species, particularly for very fine (<0·03 mm diameter) and intermediate diameter (approximately 0·19–0·29 mm) roots. Statistical analysis of the data at 29 d after sowing revealed numerous significant treatment effects for all species (not shown in Figure 1). The general response of these two root diameter classes for all species at this time was in the order: treatment C $\simeq$ treatment D > treatment B > treatment A.

Collectively, these root responses likely reflect high soil moisture stress under the clipped sward (arising from plant competition) and moderate stress under the treatment where rye was sown in the autumn (treatment B), arising from soil surface evaporation, mostly, as rye cover was only approximately 25%, compared with the herbicide treated swards, both of which had no regrowth of tall fescue during the experiment. Differences in canopy-induced competition for light under the different treatments also may have influenced shoot-to-root photosynthate allocation to seedlings, restricting root development (Shipley and Meziane, 2002).

Conclusions

Provision of high rhizobial populations to this moderately fertile, nearly neutral pH, channery loam soil resulted in nodulation of lucerne, red clover and white clover, interseeded into tall fescue. The extent of nodulation, determined by comparisons with pot-grown plants, varied with renovation technique, as did root growth. Nodulation and root growth corresponded with treatment, being higher in the herbicide-treated swards with lower plant cover. This, in conjunction with the high correlations ($r \geq 0{\cdot}85$) found between nodulation and root growth for all species under all treatments, suggests that soil moisture content, and possibly competition for light, were the limiting factors, rather than poor nodulation (early symbiosis establishment). Both physical (soil moisture content, mechanical impedance) and chemical [allelopathic effects, as proposed by Sutherland and Hoglund (1990)] explanations for the observed restriction of root growth of forage legumes, oversown into well-established, endophyte-infected tall fescue sods, are currently being sought.

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